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Microscopic and IR spectroscopic comparison of the underwater adhesives produced by germlings of the brown seaweed species *Durvillaea antarctica* and *Hormosira banksii*

Simone Dimartino[1,2,*], David M. Savory[3], Sara J. Fraser-Miller[3,4], Keith C. Gordon[3,4], A. James McQuillan[3]

[1] Department of Chemical and Process Engineering, University of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand

[2] Biomolecular Interaction Centre, University of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand

[3] Department of Chemistry, University of Otago, P.O. Box 56, Dunedin 9054, New Zealand

[4] The Dodd-Walls Centre, University of Otago, Dunedin 9054, New Zealand

* Corresponding author: simone.dimartino@canterbury.ac.nz

Abstract

Adhesives from marine organisms are often the source of inspiration for the development of glues able to create durable bonds in wet environments. In this work we investigated the adhesive secretions produced by germlings of two large seaweed species from the South Pacific, *Durvillaea antarctica*, also named "the strongest kelp in the word", and its close relative *Hormosira banksii*. The comparative analysis was based on optical and scanning electron microscopy imaging as well as FTIR spectroscopy and principal component analysis (PCA). For both species, the egg surface presents peripheral vesicles which are released soon after fertilization to discharge a primary adhesive. This is characterized by peaks representative of carbohydrate molecules. A secondary protein-based adhesive is then secreted in the early developmental stages of the germlings. EDX, FTIR and PCA indicate that *D. antarctica* secretions also contain sulphated moieties, and become cross-linked with time, both conferring strong adhesive and cohesive properties. On the other hand, *H. banksii* secretions are complemented by the putative adhesive phlorotannins, and are characterized by a simple mechanism in which all constituents are released with the same rate and with no apparent cross-linking. It is also noted that the release of adhesive materials appears to be faster and more copious in *D. antarctica* than in *H. banksii*. Overall, this study highlights that both quantity and quality of the adhesives matter in explaining the superior attachment ability of *D. antarctica*.

Keywords

Adhesive, brown algae, microscopy, ATR-FTIR, principal component analysis.

1. Introduction

Adhesive formulations and designs inspired from Nature represent the new frontier for the production of smart, green, biocompatible and sustainable adhesives and sealants. Biological adhesives can be exploited in a vast range of industries including defense, naval, biomedical, biosensor, and surgical applications [1]. For example, devices mimicking the attachment of geckos are now a reality in climbing robots [2], while biological adhesives derived from mussels are under investigation as surgical tissue adhesive [3]. For these reasons research interest in biological adhesives is receiving increasing attention by both the academic and the industrial worlds, in the search for both new "sticky" organisms and new applications [4,5].

Nature offers an extensive range of biological species with adhesive capabilities which can be sourced to seek ideas and inspiration. For example, underwater adhesion is very elegantly achieved by a multitude of organisms such as mussels [6], barnacles [7], sandcastle worms [8], sea urchins [9] and starfishes [10] to name a few. All of these species are able to firmly adhere in wet environments, either temporary or irreversibly, an achievement that humans still struggle to successfully mimic.

The attachment achieved by brown algae is particularly fascinating. For example, adult plants of the giant kelps *Macrocystis pyrifera* and *Nereocystis luetkaena* [11] and the fucoid *Durvillaea antarctica* [12] can easily be longer than 10 m and weigh more than 50 kg. They are able to produce a strong, underwater, irreversible and long-lasting attachment with a surface, able to withstand the severe hydrodynamic drags associated with wave action and tidal currents [13,14]. These features make brown algae especially interesting in the bio-adhesion arena. In particular, *Durvillaea antarctica* and *Hormosira banksii* thrive in the intertidal zone, i.e. in wave-exposed areas where the hydrodynamic drag is highest. These two species are closely related, belonging to the most evolutionally developed species within the class of the Phaeophycaceae. They display characteristic dichotomous reproduction, i.e. sexually distinct plants releasing separate male (sperm) and female (eggs) gametes, with consequent production of a zygote upon mating of the two gametes [15]. However, the two plants are distinguished, among other features, by their different location in the intertidal habitat and in their size: while *H. banksii* is relatively small, with an average size of 40 cm, and mostly present in relatively wave-sheltered locations [16,17], *D. antarctica* can be as long as 10 m and thrives only in extremely wave-exposed areas [12,18]. This observation leads to the postulate that the two species have different attachment mechanisms. Interestingly,

Stevens *et al.* suggested that *D. antarctica* might be "the strongest kelp in the world" because of its particularly high attachment strength and the mechanical properties of its thallus [19]. Taylor *et al.* carried out basic adhesion studies on brown algae zygotes aimed to explain ecological differences in gamete dispersal and zygote settlement, demonstrating that attachment of *D. antarctica* zygotes is greater than that presented by *H. banksii* even in the early stages of their life cycles [20].

The use of zygotes for the determination of the adhesion characteristics of seaweed is particularly interesting. While adult seaweeds cling onto surfaces by a combination of mechanical (interlocking with surface asperities in the mm scale) and chemical interactions, zygotes must primarily rely on chemically-based adhesion. For example, Dimartino *et al.* employed a laminar flow cell to estimate the adhesion strength of settled zygotes of *H. banksii* while excluding the mechanical component of their attachment [21]. In addition, even though gametes do not have adhesive abilities, the secretion of bio-adhesive components is immediately triggered upon fertilization to maximize survival rates in the harsh natural environment. For these reasons, zygotes represent an ideal system to further study the attachment characteristics of the glue produced by large seaweed.

Different methods have been used to determine the structural characteristics as well as the chemical composition of biological adhesives from seaweed species, including optical [22,23] and electron microscopy (SEM [24–26], ESEM [27] and TEM [23,28,29]), atomic force microscopy (AFM) [30–32], quartz crystal microbalance with dissipation (QCM-D) [33,34], zeta potential [35], energy dispersive X-ray (EDX) [29,30], chemical extraction [36,37], enzymatic assays [38,39], staining and labelling techniques [23,24,40], and infrared spectroscopy [29]. The preparation protocols as well as the testing procedure of most methods requires specific conditions often harmful to the biological sample tested and in most cases lead to the death of the specimen, hence the information obtained is usually a snapshot of the sample at a certain point in time. Among others, attenuated total reflection Fourier transform infra-red (ATR-FTIR) spectroscopy is an effective technique to study biological adhesives, probing only a thin layer of the sticky materials deposited on the optical ATR element. ATR-FTIR is particularly interesting as it is non-invasive technique that permits the analysis of biological samples in conditions resembling their natural environment, making it possible to maintain the physiological requirements necessary for cell survival. The opportunity offered by ATR-FTIR to study the production of bioadhesives from complex biological species in real-time, *in-vivo* and *in-situ* has been clearly recognized by two recent reviews by Barlow and Wahl [41] and Petrone [42]. For example ATR-FTIR has been successfully employed to

identify the composition of adhesives from a range of biological species including barnacles [43], bacteria [44], diatoms [30], algae [29], and mussels [45].

In general brown algal bioadhesives are a complex mixture of different organic compounds including proteins, carbohydrates, glycoproteins, polyhydroxyphenols and metal ions, mutually interacting through cross-linked bonds as well as electrostatic forces and metal ion bridge complexes [30,46–50]. Most of the organic compounds listed above have been associated with the adhesive simply because they are secreted after fertilization, even though the same compounds probably have other important physiological and ecological roles e.g. in the formation of the cell wall, prevention of polyspermy, detention of grazing predators, etc. For example, Bitton *et al*. demonstrated that oxidation and cross-linking mediated by a haloperoxidase enzyme is not strictly required to achieve strong adhesion bonds, questioning the role of the enzyme in algal attachment [51].

In the present work we have studied and compared the adhesion of recently fertilized zygotes of the two intertidal seaweed species *H. banksii* and *D. antarctica*, with the objective of identifying the key features that make the latter the "strongest kelp in the word". Microscopy was initially employed to clarify the mechanism of release of the glue ingredients in kelp zygotes as well as morphological changes in the adhesive pad. Energy dispersive X-ray (EDX) spectroscopy was also considered as a tool to identify specific elements in the attaching holdfast. ATR-FTIR spectroscopy was then employed to characterize the chemical constituent present in the adhesive secretions. Principal component analysis (PCA) was finally applied to the spectroscopic results to find small sources of variance in the spectral features associated with the chemical components involved in the attachment process. PCA is a widely used technique in investigative spectroscopic analysis [52–55], however at this point in time has been used minimally in the field of bioadhesives.

2. Materials and Methods

2.1. Sample collection and preparation of gamete suspensions

The procedure employed to harvest suspensions of gametes from *H. banksii* and *D. antarctica* reflects the protocol described by Dimartino *et al*. [56]. Briefly, fertile fronds from mature plants were collected at Shag Point (-45° 27' 48" , 170 ° 49' 20") in August 2013, transported in a chilly bin and thoroughly washed with 2 µm filtered and UV-treated seawater. Each frond was stored in a separate plastic bag and kept in the dark at 4 °C for a period of between 12 and 48 h, followed by thermal and light shock treatment using 2 halogen portable

floodlights (500 W each) to stimulate gamete release. Sex of the gametes released is determined by visual inspection of the exudates, i.e. white and orange for male specimen of *D. antarctica* and *H. banksii*, respectively, and olive brown for female gametes of both species. The various gametes were harvested by washing the blades in separate reservoirs containing sterile seawater at 13 °C. The suspensions obtained were filtered through plankton nets (mesh size was 105 and 25 µm for eggs and sperm, respectively) and further clarified by three subsequent sedimentation cycles under gravity. At all times, fronds, labware and suspensions were carefully manipulated to avoid anticipated contact of eggs and sperm leading to uncontrolled fertilization.

2.2. Cultures of germlings

D. antarctica and *H. banksii* zygotes were produced by simple mixing of the two gamete suspensions for around 30 min. The zygotes were inoculated onto the desired substrate and settled under gravity. The resulting cultures were placed in a temperature controlled chamber at 13 °C with 12 h:12 h light dark cycles. The light intensity during the light period was of 23 ± 6 µmol photons m^{-2} s^{-1} of photosynthetically active radiation as measured using a 2π quantum sensor coupled to a LI-250A light meter (LI-COR, Lincoln, NE, USA). Seawater was changed every 12 h to maintain the culture's viability. This protocol was effective to maintain the cultures up to at least 140 h. All due care has been devoted during sample preparation to minimize bacterial contamination. However due to the wild nature of the samples this could not be completely prevented. Yet, within the relevant time window of the experiments, the observed growth rate of the microorganisms was much less than the development of the zygotes/germlings, thus its influence on the experimental results was negligible.

In the following, the term zygote refers to fertilized eggs in the very early life stages, while germlings will be used to denote the zygotes after settling on the slide surfaces as well as at later developmental stages.

2.3. Optical, electron microscopy and energy dispersive X-ray spectroscopy

Cultures on glass and ZnSe slides (Thermo Fisher Scientific, Auckland, New Zealand, and Harrick Scientific, Horizon, Pleasantville, NY, USA, respectively) were monitored at regular intervals up to 140 h after initial settlement under optical microscopy using a Zeiss Axiostar Plus microscope equipped with a Canon Powershot A620 camera (Carl Zeiss, North Ryde, Australia). The slides were carefully removed from the seawater bath to avoid

turbulence that could potentially dislodge loosely adhered germlings. A layer of seawater at 13 °C was kept on the slides above the germlings during microscopic observation to minimize sample drying and temperature increase. A total of 4 replicate slides were observed at each time interval. Once imaged, the slides were dismissed to preclude the influence on successive germling development of environmental disturbances introduced during imaging.

Cultures on Thermanox™ coverslips (Thermo Fisher Scientific, Auckland, New Zealand) were employed for scanning electron microscopy (SEM). Four replicate slides of settled eggs as well as of germlings at 1 and 24 h following fertilization were imaged. The samples were fixed in 2.5 % gluteraldehyde in seawater, buffered in 0.1 % cacodylate and postfixed in 1 % osmium tetraoxyde (Sigma Aldrich, Castle Hill, Australia). The slides were mounted on aluminum stubs, sputter coated with gold/palladium (Emitech K575X, EM Technologies Ltd, Kent, UK) and imaged under a JEOL 6700 SEM (JEOL Ltd, Tokyo, Japan) using an accelerating voltage of 3 kV. Preliminary information on the elemental composition of the adhesive pad were obtained using the EDX system fitted in the SEM (JEOL 2300F EDS, JEOL Ltd, Tokyo, Japan) at an accelerating voltage of 10kV.

Different sample preparation methods were employed, including freeze-drying, cryo-SEM (Gatan Alto 2500 cryo stage, Gatan Inc, Pleasanton, CA, USA) and critical point drying (CPD, Bal-Tec CPD-030, Bal-Tec AG, Balzers, Liechtenstein). While all preparation methods consistently produced similar morphological features of the samples imaged, freeze drying often caused cell breakage and a barely visible adhesive footprint, cryo-SEM resulted in germling detaching from the SEM stubs, while CPD preserved the structure of the attached cells and their adhesive secretions. Accordingly, unless otherwise stated, the images presented in the mauscript will be as obtained through CPD.

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Features in the microscopy images were measured using ToupView software (v 3.5.563, ToupTek Photonics, Zhejiang, P.R.China). In the Results and Discussion section the average size of objects is reported ± standard deviation (SD).

2.4. ATR-FTIR spectroscopy

Spectroscopic measurements were carried out with a Digilab FTS-4000 FTIR spectrometer (Digilab, Marlborough, MA, USA) equipped with a KBr beamsplitter and DTGS detector and controlled by Digilab Resolutions Software (v 4.0). A Horizon ATR-FTIR optical accessory with a ZnSe 13-reflection 50 x 10 x 2 mm (45°) prism (Harrick Scientific, Pleasantville, NY, USA) was mounted in a custom built flow cell fitted in a temperature controlled jacket at 13 °C in a similar setup as depicted previously [45,57]. The penetration depth of the

exponentially decaying evanescent wave into the sample is about 0.7 μm at 1500 cm^{-1} for the ZnSe/seawater interface. All spectra were obtained from 64 co-added scans at 4 cm^{-1} resolution measured at regular time intervals.

The solutions were fed to the ATR flow cell at a flow rate of 2 ml min^{-1} using a peristaltic pump placed upstream of the ATR measuring chamber. A spectral background was acquired in an initial equilibration step with flowing seawater at 13 °C for 30 min. In the presented absorbance spectra, which may be regarded as difference spectra, the seawater absorptions in both sample and background spectra are effectively removed. After equilibration, 5 ml of the egg suspension was introduced into the flow chamber, where the female gametes settled on the ZnSe surface by gravity forming a relatively uniform monolayer of eggs. Seawater was then flowed for 1 h to allow stable settlement of the eggs on the prism surface, followed by introduction of the sperm suspension for 30 min. Finally, seawater was fed again to wash out excess of sperm and the first 24 h of germling development was spectrally monitored. It is worth noticing here that fertilization is a fast process which occurs in around 10-15 min with high success rates (> 95%) [58,59], therefore it was assumed all viable eggs settled on the prism surface were successfully fertilized.

The potential noxious effect of ZnSe solutes on the cultures was evaluated using control cultures on glass slides. The cultures were monitored up to 140 h. Cell division and germling proliferation occurred similarly on both substrates, indicating no apparent toxic effects arising from ZnSe in the early life stages of the germlings.

2.5. Principal component analysis

PCA was carried out on the ATR-IR spectra to identify small sources of variance in the spectra. The spectra were first preprocessed using standard normal variate (SNV) normalization over the spectral region of interest which was 900 to 1800 cm^{-1}. PCA was carried out on these normalized spectra with full cross validation for outlier identification. The two species were analysed both separately and together to identify variance within and between the two species. The preprocessing and PCA analysis were carried out using the Unscrambler X V13 (Camo, Norway).

3. Results and discussion

3.1 Microscopy

240 The development of *H. banksii* and *D. antarctica* germlings was compared under optical
241 microscopy (Figure 1) and SEM (Figure 2 and Figure 3) at different times.
242 Eggs, zygotes and cultured germlings from *D. antarctica* are smaller in size than those of
243 *H. banksii*. For example, the zygotes have an average diameter of 37 ± 3 μm and 66 ± 1 μm
244 for *D. antarctica* and *H. banksii*, respectively. This observation offers a first morphological
245 clue for the stronger attachment (i.e. higher survival) of *D. antarctica* germlings in wave-
246 exposed environments. In fact, accordingly to Boulbene *et al*., the hydrodynamic forces
247 experienced by a submerged spherical body scale with the cube of its radius [60]. Thus, *H.*
248 *banksii* germlings will experience hydrodynamic stresses one order of magnitude higher than
249 the ones acting on the comparatively smaller *D. antarctica* germlings.
250 Germlings from both species undergo similar growth phases, however *D. antarctica* seems
251 to develop faster than its close relative *H. banksii*. In particular, 6 h old germlings of *D.*
252 *antarctica* present apparent polarization and the formation of a rhizoidal tip (Figure 1A),
253 while the first cellular division occurs between 24 and 36 h after fertilization (Figure 1B),
254 with the differentiation of a rhizoidal mother cell and an apical mother cell. After 140 h (6
255 days), *D. antarctica* germlings have a prolonged body of variable dimensions approximately
256 90–360 μm long, i.e. 3 to 10 times the size of the original zygotes. In a few instances the
257 thallus detaches from the surface allowing the germlings to stand upright, with attachment to
258 the substrate secured through the rhizoid only (arrows in Figure 1C). On the other hand, *H.*
259 *banksii* germlings grow to a lesser extent and show first signs of polarization only after
260 around 36 h (Figure 1E). Cultures 140 h old display germlings still entirely anchored to the
261 substrate, suggesting a less mature rhizoid not able to guarantee a solid attachment to secure
262 the germling. Morphologically, these are characterized by a bulky apical cell of similar
263 dimensions as the zygote (diameter of head 71 ± 4 μm) and a thin rhizoidal prolongation 317
264 ± 39 μm long (Figure 1F). Accordingly, faster physiological development of *D. antarctica*
265 germlings can be reasonably associated with greater production of adhesive components with
266 respect to *H. banksii* germlings, a second clue to explain the stronger adhesion of the former
267 species over the latter. This hypothesis will be tested in the IR section.
268 Careful inspection of microscopic images at higher magnification reveals the presence of a
269 mucilaginous coat surrounding the germlings (Fig. S1). This film fully develops in the first 24
270 h following fertilization, reaching a maximum observed thickness in the order of 10 μm. The
271 coat appears more abundant on the rhizoidal tip, especially at later times during germling
272 development. This layer has been described as a water-rich hydrogel composed of a range of
273 broadly defined mucopolysaccharides and polyphenolic material responsible for the

germlings attachment [61]. The characteristics of such adhesive hydrogel gradually secreted by the germling will be further discussed in the IR section.

The outer surface of *H. banksii* eggs (Figure 2 A and B) presents a number of polydisperse spherical vesicles (diameter 2.4 ± 1.4 μm). The eggs are not surrounded by mucilage, mainly to facilitate interaction with and fertilization by sperms [26], therefore the protruding vesicles are unprotected in the outermost layer of the eggs.

Immediately after fertilization all vesicles must be released and a smooth cell wall is formed [62] (Figure 2 C). It is possible to observe that both germlings in the image achieve a smooth surface, with one partly covered by remnants of sperm and antheridia following the fertilization step. The release of the peripheral vesicles and formation of a smooth cell wall is a distinctive characteristic in the reproduction of brown algae [63, 64]. Tilted SEM images 1 h following fertilization do not reveal signs of adhesive materials linking the germlings to the substrate (micrograph not shown). However, 24 h after fertilization some isolated connecting threads as well as residual material on the substrate become visible (Figure 2 D). The threads could be remnants of the adhering hydrogel following the evacuation procedure, possibly indicating a fibrillar structure. Overall, the cell wall remains smooth. These observations suggest that the mucilage formed after fertilization does not leave visible residues after evacuation, indicating it is a hydrogel extremely rich in water whose spare components collapse uniformly over the germling surface, without altering the smooth appearance of the cell wall.

SEM images of *D. antarctica* show the same basic features observed on *H. banksii.* For example, *D. antarctica* eggs display an irregular surface (Figure 3A) which becomes smoother soon after fertilization due to the formation of a cell wall (Figure 3B) [63]. However, distinctive morphological differences are also apparent. In fact, the roughness of *D. antarctica* eggs is not associated with protruding vesicles, rather with peculiar crater-like structures. These may correspond to erupted vesicles as a result of sample preparation. Other SEM images for the eggs show vesicles buried under this external surface, which in this case would represent an outer layer containing the vesicles (Fig. S2). It is interesting to note that *D. antarctica* eggs are able to develop a somewhat firm adhesion to substrates even prior to fertilization [65]. Two different hypotheses can be formulated to combine the SEM observations with the preliminary stickiness of the eggs: i) the vesicles are prone to eruption and release of primary adhesive components or ii) the outer layer is made of an adhesive substance, and the windows in the shell act as passageways for incoming sperm as well as other biochemical components secreted soon after fertilization.

As early as 1 h after fertilization, the surface of *D. antarctica* germlings displays a copious number of sub-micrometric vesicles (diameter 0.26 ± 0.03 μm, Figure 3B and C). Germlings observed 96 h after fertilization still exhibit such vesicles. These vesicles are monodisperse in size and uniformly present over the entire germlings' surface, possibly containing precursors of the mucilaginous coat. *D. antarctica* germlings could have developed a strategy where adhesive vesicles are released in all directions, thus maximizing the adhesion points with the asperities of the natural substrate [66]. In contrast, *H. banksii* did not display such formations, possible indication that the production of the external mucilage is slower, or that the hydrogel is weakly bound and washed away during sample preparation. Given the prominent role of the outer mucilage in surface attachment, early adhesion of *D. antarctica* germlings seems more favored than those of *H. banksii*.

A clear adhesive footprint is already visible in the first hours after fertilization (Figure 3B). The footprint is constituted by an array of adhesive pads, possible remnants of connective material that changed appearance as a consequence of sample preparation for SEM imaging. Abundant discharge of adhesive materials continues as the germlings develop. In particular, 24 h after fertilization a layer of fibrils completely covers the rhizoidal tip and connects to the substrate, functioning as a clamp to hold the germling in place (Figure 3C and D). Similar adhesive strands are also present below the apical cell, possibly reflecting analogous chemistry and secretion mechanisms of the adhesive materials over the entire surface of the germling. This observation has also been made about 96 h old germlings (SEM not reported). Structurally, the fibrils extend radially in all directions, covering a considerable distance on the surface away from the germlings (5.9 ± 0.6 μm), with a length on the same order of magnitude as the width of the rhizoidal tip (8.9 ± 1.0 μm). Multiple secondary threads interconnect proximal radial strands, both sideways and vertically, crosslinking the main fibrils. Noticeable enlargements are present on the nodes between the primary and secondary threads, as well as on the points where the radial fibers adhere to the substrate. This characteristic helps the network sustaining loads without breakage of the internal and external connections. Small traces of adhesive materials are present further away from the main fibrous pad, similar to the adhesive remnants imaged 1 h after fertilization, suggesting a progressive expansion of the attachment pad. The overall morphology of the fibrous network resembles the structure present in spider-silk anchors, with a heterogeneous meshwork of threads rather than a homogeneous adhesive substance [67]. Such complex structure is essential to dissipate the energy associated with detachment forces and to reduce risks of crack propagation.

342

343 **3.2 Energy dispersive X-ray spectroscopy**

344 EDX was also performed on the rhizoidal tip of 24 h old germlings of *D. antarctica* (Fig.
345 S3). Traces of K, C, O, Na, S, and Ca were detected in the attachment pad. The counts in the
346 C and O peaks contain a strong contribution from the polymeric thermanox slide used to
347 support the specimens, therefore quantitative conclusions on the relative amounts of the
348 various elements identified cannot be put forth. Yet, the presence of K, Na, S and Ca is
349 characteristic of the adhesive holdfast. Sulfated polysaccharides are abundantly present in
350 carbohydrates secreted by large seaweed such as carrageenan and fucoidan [68], thus
351 explaining the abundant presence of this element in the EDX spectrum. The presence of
352 sulfated moieties will be further evidenced in the IR section. On the other hand, positive metal
353 ions contained in seawater readily form electrostatic complexes with negatively charged
354 polysaccharides and polyphenolics present in the adhesive secretions [46]. In particular, Ca
355 ions are able to form divalent coordination complexes, thus mediating both adhesion and
356 cohesion, e.g. by bridging the negatively charged adhesive secretions with a negatively
357 charged surface and by favoring gelling of the carbohydrate network [69]. Ca ions have been
358 extensively reported as key components in adhesive secretions in marine organisms, including
359 bacteria [70] and sandcastle worms [71] and will probably play a key role also in brown algal
360 attachment. The absence of a suitably developed adhesive pad in *H. banksii* germlings
361 prevented to employ EDX on this species and complete a full comparison between the two
362 species. Future work will be targeted to the analysis of the footprints of the germlings, so to
363 clarify the elemental composition of the adhesive pads and the putative role of the various
364 metal ions present.

365

366 **3.3 ATR-FTIR analysis**

367

368 **3.3.1 IR spectra from eggs before fertilization**

369 Prior to study of the adhesive produced by the settled germlings, preliminary IR
370 information on the content of the peripheral structures initially present around the eggs are
371 required. The spectra obtained from settled eggs of the two seaweed species in seawater are
372 reported in Figure 4. The absorbances are of the same order of magnitude for both spectra,
373 confirming that a relatively uniform and densely packed monolayer of eggs was produced,
374 also consistent with visual observation of the settled eggs on the ATR prism. Accordingly, the
375 amount of material sampled by the IR evanescent wave will be similar for both species, and

376 spectral differences are predominantly associated with the chemical composition of the egg
377 surface layers. These spectra can be compared qualitatively. Note that cell size may have a
378 minor influence on the sample of cellular material probed, but this effect was considered
379 negligible for the experimental system investigated.
380 Peaks in the 1000-1100 cm^{-1} region are in common for the two species, with characteristic
381 signature for polysaccharides (νC–O, and ring vibrations at 1086 cm^{-1}, νC–O–C and νC–C at
382 1063 cm^{-1}, and νC–O–C and νC–C at 1038 cm^{-1}, and νC–O–H at 1005 cm^{-1}). From the
383 intensity of the two spectra, it appears that the amount of polysaccharide compounds probed
384 by the IR is higher in eggs from *D. antarctica* than those from *H. banksii*.
385 Strong differences are remarkable at higher wavenumber regions. In particular, bands
386 characteristic for phlorotannins are apparent in the spectra from *H. banksii* eggs, with peaks at
387 1610 and 1546 cm^{-1} (C=C aromatic ring vibrations), 1208 cm^{-1} (C–O–C stretch in aryl-aryl
388 ethers) and 1154 cm^{-1} (C–O stretch in phenolic compounds) [72]. It is worth noticing that the
389 phlorotannin molecules probed in solution display a significantly different spectra than those
390 reported for solid phlorotannin extracts [73,74]. In particular, the bands comprised between
391 1450 and 1200 cm^{-1}, strong in the solid state, will have a dampened intensity in aqueous
392 environment. The difference is probably due to the formation of hydrogen bonds with water
393 molecules, thus altering the vibrational states of the polyphenols (see Fig. S4 in the
394 Supporting Information for spectra of phloroglucinol in solid state vs in aqueous solution). On
395 the other hand, *D. antarctica* eggs present a broad band in the 1199-1269 cm^{-1} range (centred
396 at around 1240 cm^{-1}), likely related to the SO_2^- asymmetric stretch in complex sulphated
397 polysaccharides such as carrageenan, porphyran and fucoidan, constituents usually
398 abundantly present in seaweed extracts from brown algae with broad absorption in the 1195-
399 1280 cm^{-1} region [30,75–77]. The width of this peak could be affected by the specific
400 configuration of the charged moiety in the polysaccharide, e.g. its position around the
401 pyranose ring [68]. Proteins are another major constituent of the outer layer of *D. antarctica*
402 eggs, with strong bands at 1651 and 1547 cm^{-1}, related to the amide I and II modes of proteins
403 in α-helix conformation [78].
404 In conclusion, the two seaweed species share only IR signatures of carbohydrate-related
405 compounds. In addition, while *H. banksii* eggs contain phlorotannin based constituents in its
406 periphery, the *D. antarctica* surface layer contains protein molecules and sulphate
407 carbohydrates. Phlorotannins have been reported to be constituents of algal adhesives [79],
408 but need to be cross-linked to achieve the optimal adhesive properties [46,80]. These are
409 likely stored in the peripheral vesicles of *H. banksii* eggs, ready to be released in the

extracellular environment upon fertilization [48,58]. Vesicles containing phlorotannins have also been reported for *D. antarctica* [81,82], but they seem to be contained within the eggs thus not probed by the IR evanescent wave [64]. Interestingly, ionic moieties present in the carbohydrate chains have a strong tendency to form stable complexes with metals and metal ions [83]. In addition, negatively charged polysaccharides are highly hygroscopic and form stable hydrogels in the presence of divalent metal ions such as Ca^{2+} and Mg^{2+} as present in seawater [84]. This mechanism is consistent with the EDX results previously presented. Accordingly, the observed tendency of *D. antarctica* eggs to bind to substrates prior to fertilization might be explained by presence of an adhesive layer composed of a fraction of partially secreted sulphated polysaccharides. The high protein component present in *D. antarctica* may be involved in the formation of the adhesive bond mediating cross-linking reactions [46,49] or simply is excreted as a result of other cellular processes.

3.3.2 IR spectra from *H. banksii* germlings

Our previous microscopic observations, as well as previous studies on the formation of the adhesive mucilage [61], indicate that a connective layer in the order of a couple of micrometres thick is being produced between the germling and a substrate following fertilization. Accordingly, we hypothesize that the substances interacting with the surface, i.e. the ones probed by the IR beam, are the ones mostly involved in the adhesion process.

In Figure 5 are presented the changes in IR spectra from *H. banksii* germlings in the 24 h following fertilization. These results are reported as difference spectra obtained by subtracting the initial ATR-IR spectrum recorded at the end of the fertilization stage. Accordingly, any spectral contribution from sperm suspension is removed in the subtraction and the spectral evolution considered is representative of germling development only. The spectra are very similar to spectra of bacterial species, with distinct bands at 1651 cm^{-1} (amide I), 1544 cm^{-1} (amide II), 1454 cm^{-1} (C–H deformation), 1400 cm^{-1} (symmetric carboxylate stretch), 1245 cm^{-1} (amide III, P=O and C–O–C stretch) [85]. Some spectral changes are also visible in the 1100–1000 cm^{-1} region characteristic of polysaccharides (C–O stretch, C–C stretch, C–O–H stretch and bend, and ring vibrations). It is important to note that these changes are only a direct consequence of the fertilization, as the spectra of the eggs before fertilization didn't change with time.

PCA analysis was carried out on spectra of *H. banksii* germlings to identify the major spectral changes in the first 24 hours after fertilization. The first principal componet (PC1) shows a trend of sample evolution over time with early timepoints in negative PC1 space

444 through to later timepoints in positive PC1 space (Figure 6a). This PC is describing 97 % of
445 the spectral variance in the sample and is the most important for looking at changes in the
446 spectra. The spectral features associated with this separation are shown in the loadings plots
447 (Figure 6b) with positive features being more dominant in samples plotting in positive PC1
448 space and negative features being more dominant in samples plotting in negative PC1 space.
449 The negative PC1 features at 1005, 1041 and 1205 cm^{-1} are stronger features in the spectra
450 collected from earlier timepoints and are consistent with the polysaccharides present on the
451 egg membrane and phenolic OH [86], respectively. The positive PC1 features at 1549 and
452 1655 cm^{-1} are more dominant in the spectra collected from later timepoints and are consistent
453 with amide II and amide I features for proteins, respectively, which are released during
454 germling development.
455 The second principal component (PC2) is only describing 1 % of the spectral variance
456 across the sample set and may not be giving valuable information based on chemical
457 differences but rather the strength of the signal and baseline shape so should not be used for
458 interpretation.
459 The features identified by PCA are futher inspected with univariate analysis methods to
460 look at the kinetic profiles in comparison to PC1. In particular, the kinetic development of the
461 release of the adhesive was quantitatively tracked by measuring the relative peak absorbance
462 of bands for key adhesive constituents, i.e. amide modes, CH deformation and $\nu_{as}SO_2^-$. The
463 absorbance maxima were normalized to 1 to facilitate the comparison with PC1 (Figure 7).
464 All bands follow the same trend as the evolution of PC1, indicating that adhesion of *H.*
465 *banksii* germlings follows a relatively simple process where all components are released with
466 same rate. This result also confirms that the univariate and multivariate approaches employed
467 result in the same observations. Interestingly, the trends follow a sigmoidal shape, with a slow
468 release of adhesive components in the first few hours, followed by a rapid increase between
469 approximately 5 to 15 h post-fertilization, and concluded by a final slow release reaching a
470 plateau at the end of the period probed. Adhesion experiments performed by Taylor *et al*. and
471 Dimartino *et al*. on *H. banksii* zygotes demonstrate that little to no adhesion is achieved in the
472 first 6 h after fertilization, while higher adhesion strength is attained after approximately 12 h
473 post fertilization [21,65]. Our FTIR results corroborate these findings from a chemical
474 perspective. In fact, out of the total adhesive components released in the first 24 h, only 20%
475 are secreted in the first 6 h while more than 50 to 60% is released at approximately 12 h after
476 fertilization.

In general, production of proteinaceous material is prevalent over other biological constituents, with bands at around 1650 and 1550 cm^{-1} typical of protein in α-helix conformation. Protein-based adhesives are often reported as key components in the adhesive secretions of a number of underwater adhesive organisms such as mussels [87], barnacles [88], or arthropods [6]. The increase of the 1245 cm^{-1} band is mainly related to amide III absorption, with a possible contribution from post-translational modified amino acids with sulfated and phosphorylated moieties. Similar post-translational modifications are in fact typical of marine adhesive proteins [89,90].

Finally, after around 18 h the system seems to have achieved a relatively stable steady state, with a considerably slower production of all adhesive materials.

3.3.3 IR spectra of *D. antarctica* germlings

In Figure 8 is presented the spectral evolution of *D. antarctica* germlings in the 24 h following fertilization. As observed in *H. banksii* germlings, the spectra for *D. antarctica* also presents features typical of biological species, with distinct bands at 1651 cm^{-1} (amide I), 1544 cm^{-1} (amide II), 1454 cm^{-1} (C–H deformation), 1400 cm^{-1} (symmetric carboxylate stretch), 1245 cm^{-1} (amide III, P=O and C–O–C stretch). In this case, the spectral changes in the 1100–1000 cm^{-1} region, characteristic of polysaccharides (C–O stretch, C–C stretch, C–O–H stretch and bend, and ring vibrations), are much more apparent.

PCA analysis was carried out on the *D. antarctica* germlings spectra to identify the major spectral changes occurring in the first 24 hours after fertilization. Like with the PCA of *H. banksii,* PC1 shows a trend of sample evolution over time with early timepoints in negative PC1 space through to later timepoints in positive PC1 space (Figure 9a). This principal component describes 84 % of the spectral variance in the sample and is the most important for looking at changes in the spectra. The spectral features associated with this separation are shown in the loadings plots (Figure 9b). The negative PC1 features at 999, 1032, 1069 (broad, shoulder) and a broad envelope between 1170 and 1290 cm^{-1} are stronger features in the spectra collected from earlier timepoints. These bands are associated with vibrational models of polysaccharides and sulphated compounds present on the eggs and prominent during the early stages after fertilization. The positive PC1 features at 1541, 1558, and 1653 cm^{-1} are more dominant in the spectra collected from later timepoints and are consistent with amide II and amide I features, respectively, indicative of protein-based secretions in the adhesive.

The second principal component (PC2) accounts for a further 10 % of explained spectral variance and separates early stage zygote growth (negative PC2) from the middle timepoints

511 (positive PC2) with later timepoints in neutral PC2 space. The spectral features associated
512 with separation in positive PC2 space include features at 1015, 1051, 1094 (polysaccharides),
513 1225 ($\nu_{as}SO_2^-$), 1250 (amide III), 1360 and 1653 (amide I) cm^{-1}. The negative separating
514 features are observed at 988, 1072, 1119 and 1184 cm^{-1}. It is clear from the loadings plot that
515 baseline variation is also described by this PC. The second PC shows temporal behaviour that
516 suggests an increase or change in band intensities in the amide III and polysaccarhide region.
517 At later times these changes are less distinct with evidence of band broadening particularly in
518 the polysaccharide region. This latter finding is not inconsistent with cross-linking which
519 creates a variety of environments (and thus wavenumbers) for these vibrational modes.
520 The normalized kinetics of PC1 together with the main bands for the adhesives secretions
521 from *D. antarctica* are reported in Figure 10. The different trends followed by the kinetic
522 profiles also suggest that *D. antarctica* produces adhesive components in a stepwise fashion.
523 In particular, carbohydrate-related molecules with absorption in the 1000-1100 cm^{-1} range are
524 released first and at a relatively higher rate. In addition, with respect to what observed with *H.*
525 *banksii*, in this case the area of the 1000-1100 cm^{-1} envelope at the end of 24 h period
526 observed is approximately tenfold higher, indicating that *D. antarctica* secretes a much larger
527 amount of polysaccharides. The band at 1245 cm^{-1} develops with similar kinetics as the
528 carbohydrate envelope, indicating this band is directly related to the carbohydrate secretions,
529 therefore assigned to sulphated moieties in polysaccharides. Protein based compounds with α-
530 helix configuration (typical peaks for amide I and II at 1645 and 1546 cm^{-1}) are released in the
531 second phase of the adhesion process. The amount of protein materials secreted at the end of
532 the 24 h absorbs with similar intensity as measured on *H. banksii* germlings. The kinetic
533 evolution of the protein-related bands does not seem to be correlated to other developing
534 bands, albeit some minor peaks are likely masked by the main spectral bands.
535 Accordingly, *D. antarctica* germlings initially secrete negatively charged carbohydrates as
536 primary adhesive, and do so in much larger absolute quantities than *H. banksii* germlings.
537 Protein-related constituents are released at later stages, as secondary adhesive and possibly
538 with cross-linking functions of the previously deposited carbohydrate network. This
539 mechanism is consistent with the microscopy results and the release of two different surface
540 vesicles with time. Proteins have been associated with the crosslinking of carbohydrates in the
541 cell wall of higher plants [91] and algae [92], usually through esterification reactions between
542 uronic acid residues and hydroxyl groups on a neighbouring polysaccharide chain, but also
543 through other more complex mechanisms [93]. The specific chemistry of the polysaccharides
544 involved in the adhesion process, and in particular the abundance of side groups such as

carboxyl or sulphate, opens the opportunity to a new variety of ester-based cross-linking modes in the adhesive hydrogel. Interestingly, all bands display a slight reduction in the production rate at the end of the 24 h period monitored, but components were still abundantly secreted in the external environment and interacting with the prism surface, overall indicating a more copious and longer release of adhesive components in *D. antarctica* than previously observed in *H. banksii*.

3.3.4 Comparison of IR spectra

The spectra from both *H. banksii* and *D. antarctica* germlings were analyzed with PCA simultaneously to find the major spectral and hence chemical differences between these two species (Figure 11). Like the individual PCA analyses, the first PC is the most important to describe the spectral variation of the samples over time (70 % of spectral variance), with earlier spectra in negative PC1 space and later time points in positive PC1 space. The negative features are observed at 1001 (ν(C-O-H)), 1034, 1042 (ν(C-O-C) and ν(CC)), 1063 (ν(C-O-C) and ν(CC)), 1111 and a broad feature ~1202 cm^{-1} (ν(C-O-C) aryl-aryl ether) which can be collectively attributed polysaccharide content of the egg/zygote before secretion of the mucoadhesives. The positively separating features are observed at 1545 (broad) and 1653 cm^{-1}, attributed to amide II and amide I, respectively, and believed to be the major component of the adhesive secretions. The second PC accounts for a further 24 % of the spectral variance, and is paramount to highlight small variations in the spectra from these two species. This PC separates *D. antartica* in positive PC2 space and *H. banskii* in negative PC2 space. The positive PC2 features, associated with *D. antarctica*, were observed at 1001, 1030, 1107 and 1246 (broad) cm^{-1}, characteristic for sulfated polysaccharides. The main negative features, associated with *H. banskii*, were observed at 1200, 1474 and 1618 cm^{-1}, indicative of polyphenolic molecules. The variance in the amide I region is difficult to interpret due to interference from water signals.

PCA suggests that the production of adhesive materials follows similar stages both in *H. banksii* and *D. antarctica*, with charged (mostly sulphated) polysaccharides initially present in the egg surface followed by a prominent production of proteins. This process indicates that the carbohydrate molecules are responsible for creating quick adhesive and cohesive bonds, followed by slower formation of stronger and more durable bonds through the protein secretions. The sulphated polysaccharides must be flexible and able to penetrate the layer of bio-fouling molecules (mostly uronic acids) ubiquitously present in marine environments [94]. The sulphated polysaccharides could be related to adhesion to the substrate through

579 absorption and formation of stable interactions with the surface. Because of the high
580 concentration of divalent metal ions in seawater, and as supported by the EDX results, it is
581 reasonable to believe that the polysaccharide matrix is initially bond through metal
582 coordination bridges which can induce gelation.
583 Still, significant differences are found in the production mechanisms of the adhesive
584 molecules. For example, carbohydrates are produced in *H. banksii* at a slower rate than in *D.*
585 *antarctica*. But the most striking difference is the amount of material produced, especially
586 with regards to the polysaccharide constituents, which are ten times more prominent in *D.*
587 *antarctica* than in *H. banksii*. This observation is in line with the microscopy results, where
588 the more rapid development of *D. antarctica* germlings is plausibly associated with a larger
589 release of adhesive components. Accordingly, it is reasonable to state that the adhesive fibrils
590 observed in the SEM are mostly composed of cross-linked non-soluble carbohydrates. On the
591 other hand, the protein component may have an important role in the direct adhesion with the
592 surface, as well as in the formation of cohesive bonds.
593
594 **4. Conclusions**
595 In the present work, the adhesive secretion from germlings of *D. antarctica* and *H. banksii*
596 have been investigated through microscopy and spectroscopy methods. It is worth noticing
597 the adhesive secretions were investigated *in-situ*, *in-vivo* and in real time, thus offering novel
598 and interesting insights in bioadhesives as well as confirming the strong capabilities of the
599 experimental methods employed. Overall, the two species display many similarities in the
600 release mechanism and in the main biological constituents of the bioadhesive. Significant
601 differences have also been highlighted, offering important clues for the understanding of the
602 higher attachment performance of the adhesive from *D. antarctica* with respect to the one
603 from *H. banksii*.
604 In general, the adhesive components are initially stored within vesicles present in the outer
605 surface of the eggs. Following fertilization, the germlings produce a secondary mucilaginous
606 adhesive through the release of new morphologically different vesicles. These are particularly
607 abundant in *D. antarctica* but not detected in *H. banksii*, probably because of a slower and
608 less copious release. The adhesive pad has a characteristic fibrillar structure, composed of an
609 heterogeneous meshwork of threads paramount to dissipate hydrodynamic and other
610 detachment forces as well as minimize crack propagation. This structure is particularly
611 complex and well established in *D. antarctica* germling even after a few hours after
612 fertilization, while it only starts appearing in *H. banksii* after at least 24 h. The difference in

613 the amount and rate of the adhesive released is further confirmed by our ATR-FTIR results,
614 and partly explains the improved adhesion of germlings from *D. antarctica* over those from
615 *H. banksii*.
616 From a chemical standpoint, PCA reveals that the key compounds shared in the adhesive
617 from the two species are polysaccharides, present in the primary eggs vesicles and initially
618 released, and proteins, secreted at later stages during germling development. However, the IR
619 spectra of *D. antarctica* germlings indicate that their carbohydrates bear sulfated moieties,
620 thought to mediate strong adhesive properties and cohesive characteristics. In addition, PCA
621 suggests that cross-linking reactions, possibly facilitated by the proteins later secreted, cure
622 the carbohydrate network, thus furthering the strength of the adhesive from *D. antarctica*. On
623 the other hand, the IR spectra of *H. banksii* adhesive is specifically characterized by
624 distinctive features for phlorotannins, a putative seaweed adhesive, but no other clues of rapid
625 cross-linking reactions or other adhesive constituents are apparent.
626 Complex polysaccharides are abundant in seaweed and fulfil many of their biological and
627 ecological functions [15], therefore it is reasonable that carbohydrate species may have a
628 strong role also in seaweed attachment. The particular role sulfated polysaccharides may
629 have in the adhesion process is consistent with the formation of stable interactions with a
630 surface and within the carbohydrate network through electrostatic and metal complex bridges.
631 Currently, the prominent opinion for seaweed based adhesives describes the proteins later
632 secreted as enzymes mediating cross-linking of the polyphenolic molecules. However, our IR
633 results indicate that polyphenols, often invoked in the adhesion of seaweed species [47,95],
634 are not of primary importance in the adhesion processes, and are more likely involved in other
635 physiological processes. Levi and Friedlander have instead proposed the protein constituents
636 have a similar structure as vitronectin, one of the glycoproteins responsible for cell-cell
637 bonding in other adhesion systems [36]. In this work, we speculate that specific enzymes
638 cross-link the carbohydrate network are also produced, leading to the cohesive strength
639 required to secure the germlings to the substrate and fostering the performance of the
640 adhesive.
641 The present work helped clarify the mechanism of release and the key biochemical features
642 of the strong adhesives produced by kelps. The results presented will help the design and
643 synthesis of biomimetic counterparts, with application in wet and complex environments such
644 as in biomedicine. In the future, molecular biology methods will be employed to assess the
645 amino acid composition and sequence of the adhesive and cross-linking proteins probed, thus
646 clarifying their role in the adhesive formulation. Further investigation on the specific

functions of the polyphenolic fraction and its possible interaction with the proteins is also of relevance and will constitute material for future research. These objectives coupled with the assessment of the attachment performances of adhesives isolated from the two brown seaweed will further support the development and ideally real-word application of permanent biomimetic adhesives.

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Competing interests

The authors have no competing interests.

Author's contributions

SD designed and coordinated the study, carried out all experiments, participated in data analysis, and wrote the manuscript; DS helped in the ATR-FTIR experiments and related data analysis; SF and KG carried out the principal component analyses and helped drafting the manuscript; JM helped data analysis and drafting the manuscript. All authors gave final approval for publication.

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Figure captions

Figure 1: Optical micrographs of cultured germlings on glass substrates. A, B and C: *D. antarctica* at 6, 30 and 140 h after fertilization, respectively. D, E and F: *H. banksii* at 6, 42 and 140 h after fertilization, respectively. The arrows indicate where the thallus detaches from the surface allowing the germlings to stand upright.

Figure 2: SEM images of *H. banksii*: A) Cluster of unfertilized eggs displaying numerous peripheral vesicles, B) Magnification of the polydisperse vesicles covering the eggs surface, C) Germings 1 h after fertilization, remnants of sperms and antheridia (jacket originally enclosing sperm) are visible on the bottom-left germling and in the background, both germling are characterized by extremely smooth surface, D) Magnification of the adhesive threads produced 24 h after fertilization.

Figure 3: SEM images of *D. antarctica*. A) Unfertilized egg having irregular surface with crater-like structures. B) Germlings 1 h after fertilization displaying smooth surface with scattered surface vesicles. An adhesive footprint composed of an array of small adhesive pads is also visible. C) Germlings 24 h after fertilization with significant development of the adhesive pad. The rhizoidal cell is covered by radial adhesive threads. Additional connective material is present underneath the apical cell. D) Magnification of the interconnected network of adhesive fibrils produced by the germlings 24 h after fertilization.

Figure 4: ATR-FTIR spectra from settled eggs of *D. antarctica* (bottom line) and *H. banksii* (top line). Background from seawater.

Figure 5: Band evolution from *H. banksii* germlings in the first 24 h after fertilization. Background is settled eggs at the end of inoculation of sperm. The temporal resolution was 20 min, lines plotted every 2 h.

Figure 6: PCA analysis of the IR spectra collected during the development of *H. banksii* germlings. Pale samples representing earlier timepoints and darker for later timepoints. (a) The scores plot represents 98 % of spectral variance. (b) The loadings plots describing the spectral features contributing to spectral variance in comparison to some representative spectra.

Figure 7: Band kinetics of developing germlings of *H. banksii* normalized to 1.

Figure 8: Band evolution in the first 24 h of *D. antarctica* germlings following fertilization with sperm. Background is settled eggs at the end of inoculation of sperm.

Figure 9: PCA analysis of the IR spectra collected during the development of *D. antarctica* germlings. Pale samples representing earlier timepoints and darker for later timepoints. (a) The scores plot represents 94 % of spectral variance. (b) The loadings plots describing the

spectral features contributing to spectral variance in comparison to some representative spectra.

Figure 10: Band kinetics of developing germlings of *D. antarctica* normalized to 1.

Figure 11: PCA analysis of IR spectra collected during the development of *D. antarctica* and *H. banksii* germlings. Pale samples representing earlier timepoints and darker for later timepoints. (a) The scores plot represents 94 % of spectral variance. (b) The loadings plots describing the spectral features contributing to spectral variance in comparison to some representative spectra.